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STUDIES ON ANTI-TUMOR IMMUNITY  
II. STUDIES ON INHIBITORY EFFECT OF  
AETEROLOGOUS SPLEEN  
CELLS ON THE GROWTH OF EHRLICH  
ASCITES TUMOR

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腫瘍免疫に関する研究

II. 免疫異種脾細胞の Ehrlich ascites tumor

の発育に対する阻止効果

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(昭和41年8月7日受付)

1963年 Stuart 等は Landschutz ascites tumor 細胞を以て家兎を免疫し、その脾細胞  $5 \times 10^8$  をマウス腹腔内に接種することにより Landschutz ascites 細胞 $10^6$  の接種を受けたマウスの60%が完全に発病から免れることを報告した。類似の実験を Ehrlich ascites tumor について実施し56.7%のマウスが同じように発病を免れ得る成績を得た。St-

uart は彼の実験成果から家兎の脾細胞が cellular antibody を形成保持する結果これが腫瘍細胞に附着し、これを崩壊せしめるものと推論している。果してこれが正しいか否かについて本報告に於ても多少論じたが以下、三、四及び五報に於て、これに触れる予定である。

**Synopsis**

Studies on inhibitory activity of lymphoid cells of sensitized or immunized animals against animal tumor cells have been conducted by many workers from 1950 onwards. Similar experimental studies were performed in our laboratory since 1959. Here I will report on the experimental results concerning the beneficial cytotoxic effect of spleen cells of immunized rabbits on the growth of Ehrlich ascites tumor and some aspects of the fundamental mechanism by which highly susceptible mice of DDS strain become endowed with a high resistance against Ehrlich ascites tumor were discussed.

**Introduction**

In 1963, Stuart reported on the experiment, in which approximately 60% of mice inoculated with living spleen cells of rabbits immunized against Landschutz ascites tumor proved insusceptible to i.p. im-

plantation of  $10^6$  intact tumor cells and survived for 90 days without any clinical manifestations. Most of the mice survived for 90 days were histologically examined, which revealed no tumor<sup>14)</sup>. He has suggested that this beneficial effect of treatment with spleen cells may be due to anti-tumor antibody carried only by intact immunized cells on their surface or interior, which enables them to adhere to tumor cells which causes eventual cell damage after *in vivo* fixation of complement.

Similar studies on inhibitory effect of lymph nodes and spleen cells from immunized or sensitized animals on malignant tumor cells have been performed from around 1950 onwards. In 1952, Ellis and Kidd<sup>2)</sup> reported on inhibition of Brown Pearce carcinoma cells by suspension of lymph nodes and spleen cells from immune hosts, and in 1956, Horn<sup>4)5)</sup> studied on cytotoxicity of various anti-sera prepared against Ehrlich ascites tumor cell components. Also in 1959, Castillanos, Ketchel and Sturgis<sup>7)</sup> reported on inhibition of tumor growth in mice by sensitized lymph nodes cells, and more recently Stuart<sup>11)</sup> studied on the effect of heterologous lymphoid cells on tumor growth in 1962.

Although many studies and experiments were conducted, the actual mechanism by which spleen cells of immunized hosts exert their cytotoxic or anti-tumor effect on malignant tumor cells in animals is not precisely known at the present time and still in need of further study. Particularly, the nature of the antigen responsible for production of cytotoxic antibody, which has been presumed to be carried by intact immunized lymphoid cells, has not yet been fully pursued. Stuart's work is of much interest in connection with an important step in the course of elucidation of such mechanism, and therefore I have done a similar study on inhibitory activity of immunized spleen cells of rabbits against Ehrlich ascites tumor in mice of DDS strain according to his method used in the study on the Landschutz ascites tumor of mice.

### Materials and Methods

**Animals:** Male mice of DDS strain weighing about 20 g. and male rabbits weighing about 3.0 Kg were used for this study.

**Tumor cells:** Ehrlich ascites tumor cells were adopted as target cells. In some experiments Yoshida sarcoma cells were used in an attempt to compare the inhibitory effect of homologous spleen cells with that of heterologous spleen cells. Tumor cells have been maintained by serial implantations from one group of mice or rats to another at appropriate intervals for more than 5 years.

**Immunization of rabbits and Preparation of immunized spleen cell suspensions:** About 10 ml of ascites fluid was collected from mice with EAC and centrifuged at 3,000 r.p.m. for 30 minutes. Supernatant was discarded and saline solution was added to the sediment to obtain a 10% cell suspension. 1.0 ml of this suspension was i.v. injected into rabbits and 2 days later 0.5 ml was again given i.v. The 3rd injection of 0.5 ml was done further 2 days thereafter and 7 days later rabbits were killed and the spleens were removed. All suspensions were prepared by grinding the spleens in a homogenizer with saline solution containing Streptomycin (1.0 mg/ml), Penicillin (1,000 units/ml) and a minute amount of heparine or reptilase (Solco Basel). These were placed in tubes and centrifuged at 3,000 r.p.m. for 15 minutes. Supernatant was discarded except for No. 84 specimen, which was tested on its effect on tumor cells in comparison with that of spleen cells. The same saline solution was added to the sedimented cells and the cell count was done to get a standard dose of  $5 \times 10^8$  spleen cells for a single injection into mice. In some experiments, suspensions of non-immunized spleen cells from rabbits or immunized spleen cells from rats were also prepared according to the same method. Groups of mice (or rats) were injected i.p. with  $10^5$

or  $10^6$  intact tumor cells and 24 to 48 hours later they received a single i.p. injection of a standard dose of spleen cells.

Sera of immunized rabbits: Sera of immunized rabbits were collected from animals immediately before they were sacrificed and pooled and stored at  $-20^{\circ}\text{C}$ . Groups of mice were challenged with  $10^5$  or  $10^6$  intact EAC cells and 24 to 48 hours later they received i.p. injections of graded doses of this pooled sera.

### Experimental Results

Exp. 1. Test on cytotoxic activity of Reptilase against growth of EAC.

To prepare spleen cell suspensions we have used saline solution containing a minute amount of Reptilase (Solco Basel). This is known as an anti-coagulant agent, the use of which was the first experience for us. In order to know whether this agent being cytotoxic for tumor cells or not, graded doses were added to EAC cells and these mixtures were kept at  $37^{\circ}\text{C}$  for an hour, and then the cells thus treated were i.p. inoculated into mice.

As shown in Table 1, we could not recognize any appreciable cytotoxic effects on tumor growth, even if a dose of as large as 4,000 mcg was applied to  $10^5$  EAC cells (Table 1).

Table 1 Cytotoxic effect of graded doses of Reptilase

Mice	EAC cell dose	Reptilase in mcg	Ascites tumor death on	Survival for 80 days.
1	$10^5$	4,000	32nd	0
2	$10^5$	4,000	25th	
3	$10^5$	2,000	21st	0
4	$10^5$	2,000	24th	
5	$10^5$	1,000	21st	0
6	$10^5$	1,000	29th	
7	$10^5$	1,000	21st	
8	$10^5$	1,000	29th	
9	$10^4$	500	21st	0
10	$10^5$	500	23rd	
11	$10^5$	500	20th	
12	$10^5$	500	18th	
13	$10^5$	250	31st	0
14	$10^5$	250	16th	
15	$10^5$	125	33rd	0
16	$10^5$	125	21st	

Exp. 2. Test on inhibitory effect of spleen cells of immunized rabbits on tumor growth of EAC.

Spleen cells were collected from total 6 immunized rabbits and cell suspensions were prepared as described previously. Total 30 mice of the test group and total 27 mice of the control group were inoculated i.p. with  $10^6$  intact EAC cells and 24 hours later mice of the test group received a single i.p. injection of  $5 \times 10^8$  spleen cells, while mice of the control group received nothing else. The onset of ascites was judged by clinical appearance or by a weight increase of 5 g. in 2 days. Control mice all died during the course of 15 to 40 days. At autopsy the peritoneal cavity was filled with turbid ascites fluid which sometimes contained masses of small tumors. Invasion of viscera was rarely seen at the later stage of mice survived for a longer time. When given the tumor cells at the level of  $10^6$  dose and treated 24 hours later with  $5 \times 10^8$  immunized spleen cells, 17 out of 30 mice survived for more than 80 days and detailed postmortem examination failed to reveal tumor (Table 2). Other 2 mice survived up to the 78th and 80th day respec

Table 2 Cytotoxic effect of immunized spleen cells on Ehrlich ascites tumor

Rabbit No.	Dose of spleen cells	Chal. dose of EAC cells	No. of test mice	Mice died from EAC	Mice suffered but survived for 80 days	Mice not suffered
No. 84	$5 \times 10^8$	$10^6$	2 *	0	0	2
No. 97	$5 \times 10^8$	$10^6$	4	1	0	2
No. 76	$5 \times 10^8$	$10^6$	8	3	1	4
No. 41	$5 \times 10^8$	$10^6$	5	2	1	3
No. 42	$5 \times 10^8$	$10^6$	3	2	0	1
No. 33	$5 \times 10^8$	$10^6$	8	3	0	5
Total			30	11	2	17 (56.7)
Control groups						
No. 84	/	$10^6$	4	4	0	0
No. 97	/	$10^6$	5	5	0	0
No. 76	/	$10^6$	5	5	0	0
No. 41	/	$10^6$	4	4	0	0
No. 42	/	$10^6$	4	4	0	0
No. 33	/	$10^6$	5	5	0	0
Total			27	27	0	0

\* Other 2 mice were treated with supernatant of spleen cell suspension, without effect.

\*\* 3 out of 5 mice were rechallenged on the 42nd day, but well tolerated.

tively, when they were killed and histologically examined. They had solid tumors instead of ascites tumor in the peritoneal cavities. These mice of completely survived group were rechallenged i.p. with  $10^6$  EAC cells on the 42nd day, but they all well tolerated this second implantation of the tumor cells and survived up to the 84th day, when sacrificed and histologically examined. No characteristic findings were revealed by this examination. Thus the complete survival rate was 56.7%, which was almost the same as that of Stuart's experiment with Landschutz ascites tumor. We did not observe tumor versus host disease in the test mice, presumably because, as Stuart has suggested, these spleen cells survive for only a short time in the recipient.

Exp. 3. Test on inhibitory effect of spleen cells of non-immunized rabbits on growth of EAC.

Spleen cell suspensions were prepared from non-immunized rabbits. Total 10 mice were i.p. inoculated with  $10^6$  intact tumor cells and 24 hours later they received a single i.p. injection with  $5 \times 10^8$  non-immunized spleen cells. No specially beneficial inhibitory effect on tumor growth was observed and all mice developed ascites tumor just like control mice and succumbed within 20 to 40 days. It is clear that spleen cells must be immunized against EAC cells if they are to exert inhibitory action against target tumor cells.

Since spleen cells of non-immunized rabbits could contribute little, if any, to inhibition of tumor cell growth in the recipient, it seems likely that such rabbits would be, at least, immunologically susceptible to that tumor if no other special mechanisms are available.

Exp. 4. Test on inhibitory activity of sera of immunized rabbits against EAC.

Pooled sera stored at  $-20^\circ\text{C}$  were tested on inhibitory activity against tumor growth in the recipient. Groups of mice were i.p. inoculated with  $10^6$  intact tumor cells and 24 hours later they received sera.

in graded doses of 0.5 ml, 0.3 ml, 0.1 ml and 0.05 ml intraperitoneally. This treatment proved of no appreciable effect on tumor growth and each 3 mice of total 4 groups developed ascites tumor and died between the 15th and 28th day.

From these data, it seems likely that circulating antibody of immunized rabbits can contribute little, if any, to inhibition of tumor growth in mice.

Exp. 5. Test on inhibitory effect of homologous spleen cells of immunized rats on growth of Yoshida sarcoma.

Rats were i.p. inoculated with  $10^6$  Yoshida sarcoma cells and then treated with i.p. injections of Marinamycin<sup>6)</sup> and Soedomycin (reported in previous report) starting 24 hours after inoculation of tumor cells. Some rats were rescued from developing ascites tumor. Most of such rats proved capable of resisting against reimplantation of  $10^5$  and  $10^7$  intact tumor cells at intervals of 2 and 3 weeks and survived for more than 80 days without any clinical manifestations. From such rats spleen cell suspensions were prepared according to the same method as used for preparation of rabbit spleen cell suspensions.

Normal rats were given i.p.  $5 \times 10^8$  spleen cells of immunized rats and 5 days later they received a dose of  $10^6$  Yoshida sarcoma cells intraperitoneally. Six out of 7 rats well tolerated this cell implantation and survived up to the 52nd day, when they were sacrificed and histologically examined, which failed to reveal any findings characteristic of tumor (Table 3).

Table 3 Cytotoxic effect of homologous spleen cells of rats made immune against Yoshida sarcoma

Test rats No.	Rats No. made immune	Spleen cell dose	Challenge dose of YS cell	2nd chal. on 21st day	3rd chal. on 33rd. day	Postmortem 52nd day
1	No. 19— 2	$5 \times 10^8$	$10^6$ (Nil)	$10^6$ (Nil)	$10^7$ (Nil)	Nil
2	No. 43— 1	$5 \times 10^8$	$10^6$ (Nil)	$10^6$ (Nil)	$10^7$ (Nil)	Nil
3	No. 43— 1	$5 \times 10^8$	$10^6$ (Nil)	$10^6$ (Nil)	$10^7$ (Nil)	Nil
4	No. 33— 1	$5 \times 10^8$	$10^6$ (Nil)	$10^6$ (Nil)	$10^7$ (Nil)	Nil
5	No. 33— 1	$5 \times 10^8$	$10^6$ (Nil)	$10^6$ (Nil)	$10^7$ (Nil)	Nil
6	No. 33— 1	$5 \times 10^8$	$10^6$ dead	/		
7	No. 33— 1	$5 \times 10^8$	$10^6$ (Nil)	$10^6$ (Nil)	$10^7$ (Nil)	Nil
Total dead: survived ( 1 : 6 )						
8		Control	group			
		/	$10^6$ dead (10th)			
9		/	$10^6$ dead (14th)			
10		/	$10^6$ dead (12th)			
11		/	/	$10^6$ dead (10th)		
12		/	/	$10^6$ dead ( 9th)		
13		/	/	$10^6$ dead (12th)		
14		/	/	/	$10^7$ dead ( 7td:)	
15		/	/	/	$10^7$ dead (11th)	
10		/	/	/	$10^7$ dead ( 8th)	
Total dead : survived ( 9 : 0 )						

### Discussion

Stuart reported on experimental studies on beneficial inhibitory effect of spleen cells from immunized

rabbits on growth of Landschutz ascites tumor of mice, at the 3rd International Congress of Chemotherapy held at Stuttgart in 1963. Similar results were obtained from our experimental studies on cytotoxic activity of spleen cells of immunized rabbits against ascites cells of Ehrlich ascites tumor. About 60% of the mice treated with a single i.p. injection of  $5 \times 10^8$  immunized spleen cells proved to be almost completely insusceptible to a challenge dose of  $10^6$  intact tumor cells. This result was almost the same as that of Stuart's work with Landschutz ascites tumor of mice. Since spleen cells obtained from non-immunized rabbits could contribute little, if any, to inhibition of tumor growth in the recipient, it seems likely that spleen cells must be immunized if they are to exert such cytotoxic action, and that natural anti-tumor resistance seen in insusceptible animal species cannot be explained as a result of anti-tumor antibody naturally inherent in spleen cells of such animals. Spleen cells of non-immunized rabbits lack antibody against foreign tumor cells, so that such animals would be, at least immunologically, susceptible to tumors of foreign animal species, provided no other special mechanisms are available.

Spleen cells of naturally insusceptible animal species may inherit a higher immunological competence to respond to stimulation of foreign tumor antigen than that inherent in the cells of susceptible animal species. Thus spleen cells of the former species may respond to foreign tumor antigen with rapidity and ease, and as a result sufficient lymphoid cells immunized against tumor cells may be produced to suppress tumor growth and eventually destroy all of the tumor cells. At this stage, however, anti-tumor resistance of such animals can be explained as an acquired type of immunity against tumor cells.

Since the sera of immunized rabbits proved incapable of exerting inhibitory effect on tumor growth, circulating antibody seemed to be able to contribute little to inhibition of tumor growth.

Homologous spleen cells of immunized rats were capable of exerting a similar beneficial cytotoxic effect on Yoshida sarcoma cells. In spite of the fact that spleen cells were injected into rats 5 days before challenge with intact tumor cells, a complete survival rate of rats seemed higher than that achieved in the test with Ehrlich ascites tumor of mice. Wigzell (1961)<sup>10)</sup> did not observe any striking therapeutic effect on mammary tumor of mice with homologous spleen cells. This striking difference between both results cannot be properly explained at the present time. As will be reported in the next paper, homologous spleen cells of mice made immune against EAC or Sarcoma 180 proved capable of exerting a similar beneficial inhibitory effect on growth of respective tumors. The most possible explanation at moment may be that this difference may be due either to the different properties inherent in respective tumors or to variable immunological competence of spleen cells of different strains of animals.

As Stuart has suggested, the beneficial cytotoxic effect of immunized spleen cells may be due to anti-tumor antibody carried by them on their surface, which enables them to adhere to tumor cells which causes cell damage after in vivo fixation of complement. To search for antigenic substance responsible for production of such cellular antibody, experimental studies have been performed with various cell components by several workers, but almost no success was achieved in this field. In the previous paper, I had discussed some aspects of such antigenic substance based on our experimental evidences obtained since 1959, however, many other problems are still in need of further study.

### Summary and Conclusion

1) Mice were inoculated with  $10^6$  intact Ehrlich ascites tumor cells and 24 hours later they were treated with a single i.p. injection of  $5 \times 10^8$  spleen cells of immunized rabbits. 17 out of total 30 mice thus treat-

ed survived without clinical signs of EAC for more than 80 days. Their postmortem histological examination failed to reveal characteristic findings of the disease. Two other mice also survived up to 78th and 80th day, when they were sacrificed and histologically examined, which revealed tumors in the peritoneal cavities. The complete survival rate was 57.6%, which was almost the same as that of Stuart's study on the Landschutz ascites tumor (60%).

2) Similar test was done with spleen cells of non-immunized rabbits. We did not observe any beneficial therapeutic effect on tumor growth with nonimmunized spleen cells.

3) Sera of immunized rabbits were tested by similar method, which proved to contribute little to inhibition of tumor growth.

4) Homologous spleen cells of rats made immune against Yoshida sarcoma were tested by the same method. Rats were i.p. inoculated with  $5 \times 10^6$  immunized spleen cells and 5 days later they received a single injection of  $10^6$  intact sarcoma cells. Six out of 7 rats thus treated survived without any signs of ascites tumor up to the 52nd day, when they were killed and histologically examined, but this failed to reveal tumor. The complete survival rate of treated rats was more than 80%, which was considerably higher than that achieved in experiments with heterologous spleen cells immunized against EAC of mice.

From these experimental evidences, it seems reasonable to consider that both heterologous and homologous spleen cells must be immunized against target tumor cells if they are to exert beneficial inhibitory action against target tumors, and that this beneficial action may be due to cellular antibody carried by immunized spleen cells on their surface, which enables them to adhere to tumor cells as the first step for tumor cell damage.

There seems to exist no essential differences in the fundamental mechanism by which heterologous or homologous spleen cells of immunized animals exert their beneficial inhibitory action against tumor growth in the recipient, which will be further discussed in the next report on experimental studies on homologous spleen cells.

As I have pointed out in the previous paper, the antigenic substance mainly responsible for formation of cellular antibody should be regarded as some agent (Carcinogenic agent, Soeda) present both intra- and extra-cellular fluid of ascites tumor cells and immunological response of lymphoid cells may be rather directed toward this agent, than toward cellular constituents of tumor cells.

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